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Aneuploidy, a change in a cells karyotype involving loss or gain of chromosomes, is a key event in the etiology of breast cancer. Since aneuploidy can result from mitotic non-disjunction, understanding the process of chromosome segregation is greatly important.

The purpose of this research has been to identify new proteins that are required for maintaining the fidelity of chromosome segregation. Using budding yeast as an experimental system, we identified strong genetic interactions between *RAD23*, *DDI1*, *ESP1* and the anaphase regulator *PDS1*. We determined that Rad23p and Ddi1p are essential for a Pds1p-dependent checkpoint system that couples replication with mitosis: mutant *rad23 ddi1* cells become aneuploid when challenged with replication inhibitors. We therefore identified 2 proteins required for the regulation of chromosome segregation in yeast. Both *RAD23* and *DDI1* are highly conserved in human cells, though defects in these genes have not been previously implicated in the generation of aneuploid cells.

Knowledge such as this will allow us to define genetic and environmental factors which cause non-disjunction; many of these factors will be critically involved in the etiology of breast cancer.

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Duane Clarke 6/1/98

PI - Signature Date

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Introduction

Subject and purpose of research

Aneuploidy is a change in a cells karyotype which involves loss or gain of part of the genome. It is a prevalent genetic affliction and is a significant factor in malignancies including those of the breast; aneuploidy is a key event in the etiology of breast cancer and proliferative breast disease. Chromosome losses and gains in mammary gland epithelial cells contribute to the development of breast tumors. (1,2). Since aneuploidy can result from mitotic non-disjunction, understanding the process of chromosome segregation is greatly important. This knowledge will allow us to define genetic and environmental factors which cause non-disjunction; many of these factors will be critically involved in the etiology of breast cancer.

Equal division of a replicated genome requires that sister chromatids migrate to opposite poles of the mitotic spindle during anaphase: segregation fidelity relies on elaborate mechanics, precisely regulated by cell cycle checkpoint controls. The purpose of this research has been to identify new proteins that are required for this regulation. Since proteins involved in the process of chromosome segregation have a high degree of evolutionary conservation, we set out to use powerful genetic approaches in yeast to rapidly identify the important proteins. In this report we describe the identification of two genes which are required for regulated chromosome segregation: these genes have very closely related human homologues. It follows that defects in these proteins may cause reduced segregation efficiency and therefore increase the frequency of non-disjunction in dividing cells of the breast epithelium. An ability to recognize such defects would have a vast impact on our capacity to identify individuals prone to acquiring aneuploid cells.

Scope of research

Recent evidence strongly predicts that cell cycle regulated sister chromatid separation involves the ubiquitin-dependent degradation of an anaphase inhibitor, known as Pds1p in *Saccharomyces cerevisiae*.(3,4; see Fig1). During an unpurturbed cell cycle, Pds1p becomes poly-ubiquitinated at the metaphase to anaphase transition by multi-enzyme APC/cyclosome complexes; the modified forms are then recognized and degraded by 26S proteasome particles (4). Pds1p degradation is thought to initiate displacement of chromatid cohesion proteins, thus allowing sister separation and the onset of anaphase.

PDS1 mutants fail to execute checkpoint control in response to spindle poisons, DNA damage (3) or the replication inhibitor hydroxyurea (reported here for the first time). Checkpoint arrest in metaphase is thought to be mediated through stabilization of Pds1p. Although Pds1p is clearly a key component of the anaphase-checkpoint machinery, how Pds1p stability is regulated is not known. The ubiquitin-ligase complex (APC) which ubiquitinates Pds1p, is composed in part of Cdc23p and Cdc16p (5). Thus, *cdc23-1* and *cdc16-1* temperature-sensitive alleles confer a metaphase arrest.

Clearly, Pds1p and APC components are critically involved in the regulation of anaphase. Important questions are how proteasome and APC regulation contribute to Pds1p stability in response to checkpoints, and what are the upstream elements which mediate checkpoint signals, since failure in these controls causes aneuploidy (3). This research aimed to identify new proteins which interact with proteins known to be required for segregation fidelity (Cdc16p, Cdc23p and Pds1p). Our goal has been to characterize new proteins which function to maintain the fidelity of chromosome segregation. We have screened for genes which interact genetically with *CDC23*, *CDC16* and *PDS1*.

Several divergent approaches were used to maximize the efficiency of screening.

(i) Screen for mutations which over-ride the metaphase arrest of *cdc23-1*

(ii) Screen for mutations which enhance or suppress the temperature sensitivity of APC mutants (*cdc23-1* or *cdc16-1*)

(iii) Screen for dosage suppressors of *cdc16-1*

(iv) Yeast genome data base searches for proteins with cyclin-like destruction boxes

(v) Screen for dosage suppressors of a temperature sensitive *PDS1* allele, *pds1-128*

Background of previous work

At the onset of my DOD Breast Cancer Research Fellowship I was involved in designing genetic screens which would lead to the isolation of new proteins required for the regulated segregation of sister chromatids. Additionally, I had begun to characterize several mutants isolated from our preliminary screening attempts: 2 mutants (*dam1* and *cst1*) had a possible role in chromosome segregation, and a 3rd mutant was a novel temperature sensitive allele of the anaphase inhibitor *PDS1*.

By screening the yeast genome data base, *DAM1* was identified as a protein with a putative mitotic destruction box (similar to the destruction box of Pds1p and mitotic cyclins which is required for their degradation). A mutant allele, *dam1-19*, had been isolated from the same genetic screen from which *pds1-128* was found, making study of this protein very interesting. Preliminary data showed that Dam1p is degraded as cells exit mitosis. We had constructed a strain deleted for *DAM1*, and found a severe growth defect. Further work would characterize the mutant phenotype in detail and investigate the function of the putative

destruction box sequence.

cst1-1 was isolated from a screen for mutations which override the metaphase arrest of *cdc23-1*. Typically, over 40% of the cells arrest in telophase at the non-permissive temperature, indicating either that segregation proceeds despite the absence of Cdc23p activity or that the period from anaphase to telophase is significantly prolonged at the permissive-temperature. Outcrossing the *cst1-1* allele from *cdc23-1* into wild type background, revealed that cst1 is itself temperature-sensitive. The temperature-sensitive phenotype of *cst1* was rescued by transformation with a yeast genomic library (YCp50). The ORF which complements the *cst1* mutation is that of an uncharacterized protein.

A screen designed to find mutations that are lethal in combination with cyclin *CLB2* overexpression yielded a new temperature sensitive allele of *PDS1, pds1-128.* The previously described temperature sensitive allele of *PDS1*, *pds1-1*, was essentially a deletion, and therefore unlikely to be useful for the isolation of dosage suppressors. Additionally, we found that *pds1-128* causes a remarkable sensitivity to the replication inhibitor hydroxyurea, apparently not the case for *pds1-1.* This gave us a unique opportunity to screen for new proteins which interact genetically with *PDS1.*

Body of 1998 annual report

1. Results

Genetic screens for identifying components of a cellular system can be rapid and fruitful. Often for simple technical reasons, particular screens are not successful. Thus to maximize our gains, we initially undertook the conservative approach of employing several diverse screening strategies. For each procedure, pilot experiments were performed within the first 2 months of the fellowship. Although this proved labor intensive, we were able to quickly dismiss several options as being non-informative (described below), enabling us to specifically target efforts towards the most rewarding screens. This approach also permits continuous adjustment of our strategies in the context of a fast paced field. We have identified 2 novel suppressors of Pds1p which are important regulators of chromosome segregation. These proteins are highly conserved in humans. In light of the importance of these findings we chose to concentrate on characterizing these regulators and, if proven necessary, continue screening at a later stage.

1.1 Screens for Anaphase Promoting Complex interactors

1.1a Screen for mutations which override the metaphase arrest of cdc23-1

As described in the proposal, a screen for mutations overiding the metaphase arrest of a *cdc23*^{ts} allele was performed and initial characterization of the mutants was carried out by establishing complementation groups and the terminal phenotype of the different mutant classes at the restrictive temperature. However, detailed characterization of the tightest mutants, including *cst1*, indicated that the loci identified could not be primarily involved in sister chromatid cohesion. Therefore, judging from this pilot screening attempt, the potential for finding proteins involved in regulating sister chromatid cohesion

using this specific method may be limited. In the course of these studies, several other laboratories described alternate screening methods (carried out to saturation) which led to the identification of a putative cohesion protein (Mcd1p/Scc1p; refs 6,7).

1.1b Screens for dosage suppressors of APC mutants.

As pilot experiments, about 20,000 high copy plasmids were tested for their ability to enhance or suppress *cdc16-1* or *cdc23-1*. No novel genes were identified using this approach.

1.1c Screens for mutational enhancers/suppressors of cdc16-1

For isolating mutational enhancers/suppressors of *cdc16-1* about 20,000 mutagenized clones were initially analysed. A single supressor of *cdc16-1* was isolated (*scs1-1*). The *scs1-1* allele was outcrossed from *cdc16-1* and found to be temperature sensitive. This enabled isolation of a plasmid which recues *scs1-1*. When the *cdc16-1 scs1-1* strain was transformed with this plasmid, the original temperature sensitivity of *cdc16-1* was restored. This plasmid is 18kb and is currently been subcloned to determine the relevant ORF. The terminal phenotype of *scs1-1* grown at the restrictive temperature is also being analysed.

1.1d Genome data base searches for proteins with cyclin-like destruction boxes

Detailed genome searches for proteins with cyclin-like destruction boxes yielded a short list of proteins likely to be involved in the regulation of mitosis. One protein of unknown function had a particularly good destruction box sequence. Coincidentally, a mutant allele of this gene, *dam1-19*, had been isolated in our lab. from the same genetic screen from which *pds1-128* was found, Pds1p being an inhibitor of anaphase in yeast. We analysed *dam1-19* and a strain deleted for *DAM1*, and found a defect in cell separation following nuclear division,

suggesting a role for Dam1p in exit from mitosis. However, staining of tubulin in these cells revealed G1 spindle configurations (not shown); therefore the defect is not in mitotic exit, but rather in cell separation only. A putative function of the cyclin-like destruction box motifs was ruled out by generating mutant forms of *DAM1* which lack either one or both of the boxes: deletion of these sequences caused no obvious growth defects. Overexpression of the mutant forms resulted in no observable phenotype. These studies revealed that Dam1p has no role in controlling mitotic progression. Following these studies we showed that *DAM1* is isogenic to *SEC5*, mutations in which cause secretion defects. These findings were reported in Mondesert et al. (1997) *Genetics* v147(2):421-434.

1.2 Screen for Pds1p (anaphase inhibitor) interactors

A temperature sensitive *pds1* mutant, *pds1-128*, was isolated in a genetic screen designed to find mutations which cause lethality when *CLB2* is overexpressed (8). Since its initial isolation, it has become clear that *PDS1* is a pivotal element in the regulation of sister chromatid adhesion. Although Pds1p is a key regulator of chromosome segregation, little is known about elements which regulate Pds1p. We decided to use this allele to isolate dosage suppressors of *pds1*. At the time our position was unique since the previously described *pds1* mutants (3) were essentially null alleles. Our approach proved rewarding for two main reasons: (1) a novel checkpoint system responsive to chronic perturbations in cell cycle progression was discovered, (2) upstream regulators of *PDS1* were identified - Rad23p and Ddi1p - which are required for Pds1p-dependent checkpoints and are essential for chromosome segregation fidelity in certain checkpoint situations. This work, representing the most significant advancements made from May 1997-May 1998, is described in detail below.

1.2a Dosage suppressor screen

To identify proteins which interact with Pds1p, we screened for genes

whose increased dosage suppress the lethality of *pds1-128* grown at 37°C. From a genomic *Saccharomyces cerevisiae* library (maintained at high copy in a yeast episomal vector), 3 dosage suppressors of *pds1-128* were identified: (i) YEp-ESP1 (13 independent isolates, representing 6 plasmid types), (ii) YEp-RAD23 (4 isolates, 1 plasmid type) and (iii) YEp-DDI1 (8 isolates, 2 plasmid types). YEp-PDS1 was isolated at least 13 times. The screen is near saturation: in excess of 160,000 independent transformants were analysed and multiple plasmids were isolated representing each suppressor.

An important question is whether the suppressor effects of these genes are dependent on the presence of Pds1 protein: we constructed strains deleted for *PDS1* (*pds1Δ*) and harboring either YEp-DDI1, YEp-RAD23 or YEp-ESP1 (Fig. 2a). *pds1Δ* cells are viable in the BF264-15DU genetic background, but were temperature sensitive at 28°C on YEPD plates. Neither YEp-DDI1 nor YEp-RAD23 could rescue *pds1Δ* at 28°C (integrated versions of *RAD23* and *DDI1* induced from the *GAL1* promoter also failed to rescue *pds1Δ*, but did rescue *pds1-128*). Thus, high dosage of *DDI1* and *RAD23* cannot bypass the *pds1Δ* temperature sensitivity; these proteins must act in the same pathway as Pds1p, possibly by directly increasing Pds1p stability.

YEp-ESP1 allowed *pds1Δ* to grow up to 34°C. Therefore an increased dosage of *ESP1* is able to bypass the need for Pds1p at intermediate temperatures. A genetic interaction between *ESP1* and *pds1* was previously reported (4), and the homologous proteins in S. pombe (Cut1 and Cut2) interact physically (9).

To establish the relevance of $pds1^{ts}$ suppression by high dosage *RAD23/DDI1*, we investigated possible genetic interactions between *RAD23/DDI1* and *esp1* mutants. The biological relevance of the *ESP1/PDS1* genetic interaction is that Pds1p binds to Esp1p and thereby inhibits the anaphase-promoting activity of Esp1p. If Rad23p/Ddi1p are specific regulators of Pds1p, a genetic interaction with *esp1* should be detectable. Figure 2b shows that the lethality of strains with

temperature sensitive *esp1* alleles (*esp1-N5* and *esp1-B7*) is greatly enhanced by over-expression of *RAD23* or *DDI1*. Visualization of spindles (see experimental procedures) revealed that *RAD23* or *DDI1* overexpression caused the *esp1* mutants to display the characteristic terminal phenotype at the new restrictive temperature, confirming the specificity of the interaction (not shown).

1.2b RAD23 and DDI1 are structurally similar

The proposal that Rad23p and Ddi1p regulate Pds1p stability has several attractions. Both genes are induced by an identical UAS which is responsive to DNA damaging agents and hydroxyurea (10), as might be expected for genes involved in checkpoint control. A more striking observation is that Rad23p and Ddi1p both have UBA domains at their extreme C-termini (Rad23p has a second internally located UBA, Fig. 2c). The function of UBA domains is not known, though they are present in different classes of enzyme involved in ubiquitin-dependent proteolysis (11); an intriguing coincidence given the dependence of Pds1p proteolysis on the ubiquitin system. Rad23p and Ddi1p also share ubiquitin-like domains towards their N-termini: the ubiquitin-like domain of Rad23p was recently shown to be required for a physical interaction with 26S proteasomes (12). Whether Ddi1p binds to the proteasome remains to be tested.

1.2c pds1 and rad23 ddi1 mutants are sensitive to replication inhibition

Our genetic data suggested that Rad23p and Ddi1p have a function in the Pds1p-dependent anaphase-checkpoint pathway. We predicted that *rad23* and *ddi1* mutants should be sensitive to at least a subset of the agents to which *pds1-128* is sensitive (data summarized in Table 1). The similar structure of *RAD23* and *DDI1* led us to suspect that either protein may be sufficient for checkpoint activation, thus we constructed strains deleted for *RAD23* or *DDI1* or both.

As reported for other *pds1* mutants (3), we found that *pds1-128* and *pds1Δ*

are sensitive to nocodazole (3 h treatment with 15μg/ml; not shown) and gamma irradiation (50-200 Gy delivered at 3.6 Gy/min; not shown), but not sensitive to ultraviolet light (not shown). However, *pds1-128* and *pds1Δ* are exquisitely sensitive to the replication inhibitor hydroxyurea in the BF264-15DU genetic background (Fig. 3); apparently not the case in the background described by Yamamoto et al. (1996a). These experiments suggest that Pds1p is required for the spindle and DNA damage checkpoint, but also has an unsuspected role in checkpoint controls normally invoked by inhibition of replication.

Next we assayed strains deleted for *RAD23* or *DDI1*: our data were as expected in light of previous reports. *ddi1Δ* cells were not sensitive to any of the agents tested (in agreement with Liu & Xiao 1997). *rad23Δ* were not sensitive to nocodazole nor to gamma irradiation (50-200 Gy delivered at 3.6 Gy/min; not shown), but were sensitive to ultraviolet light, as previously described (13), and somewhat sensitive to hydroxyurea (see below).

Importantly, *rad23Δ ddi1Δ* strains revealed crucial information when tested for sensitivity to these agents. Sensitivity to gamma irradiation (50-200 Gy delivered at 3.6 Gy/min) and nocodazole (3 h treatment with 15μg/ml) were wild type (not shown). Sensitivity to ultraviolet light was identical to *rad23Δ* (not shown). However, a strong sensitivity to hydroxyurea, equal to that of *pds1-128*, was found (Fig. 3).

Hydroxyurea is the only agent equally lethal to *pds1-128* and *rad23Δ ddi1Δ*: Rad23p and Ddi1p presumably have an important role in responding to inhibition of replication. These proteins share a redundant function in this pathway since deletion of both genes causes a far more severe phenotype than deletion of either one gene by itself. The hydroxyurea sensitivity is likely to reflect a replication checkpoint defect, rather than a DNA damage checkpoint defect since *rad9Δ* cells, in which the DNA damage checkpoint is absent, are not sensitive to hydroxyurea.

1.2d pds1-128 and rad23Δ ddi1Δ strains are checkpoint defective

Microscopic analysis of *pds1-128* and *rad23Δ ddi1Δ* growing on solid media containing hydroxyurea revealed the formation of microcolonies, indicative of a checkpoint defect (Fig. 4a,b). Loss of checkpoint controls which normally respond to replication inhibition can be unequivocally demonstrated by measuring the time interval between budding and the onset of anaphase (assessed here by monitoring spindle elongation). We synchronized cells in G1 with α factor then released in to liquid media containing 0.1M hydroxyurea (Fig. 4c). Wild type cells budded after approximately 50 minutes and G2 spindles formed at 60-120 minutes; spindle elongation began 120 minutes later. In the case *of pds1-128* and *rad23Δddi1Δ*, budding and G2 spindle formation occurred at the same time as in wild type cells, but spindle elongation was advanced by 40 minutes. FACScan analysis showed that replication, budding and G2 spindle formation were coupled normally in all three strains, and that the length of S phase was similar in each case (not shown). This data confirms loss of coordination between spindle elongation and replication in the mutants.

1.2e Sensitivity of pds1-128 to hydroxyurea is suppressed by RAD23 or DDI1 overexpression.

If *RAD23* and *DDI1* have a specific role in the Pds1p-dependent checkpoint system which responds to replication inhibition, overexpression of *RAD23/DDI1* should suppress the sensitivity of *pds1-128* to hydroxyurea. This is indeed the case: *pds1-128 GAL1:RAD23* or *pds1-128 GAL1:DDI1* strains cannot grow on YEPD plates containing 0.1M hydroxyurea (*GAL1:RAD23/GAL1:DDI1* repressed), but grow similar to wild type on YEPG plates containing 0.1M hydroxyurea (*GAL1:RAD23/GAL1:DDI1* induced) (Fig. 5). Crucially, *GAL1:RAD23* and *GAL1:DDI1* cannot rescue the hydroxyurea sensitivity of *pds1Δ* strains, again revealing that rescue is dependent on the presence of Pds1 protein.

Given the above data, we predicted that overexpressing *RAD23* or *DDI1* should delay anaphase in the presence of hydroxyurea. Cells were synchronized with α factor and released in to hydroxyurea-containing media with a galactose carbon source to induce expression of *GAL1:RAD23* or *GAL1:DDI1*. As expected the interval between budding and anaphase was consistently greater than in control cells (Fig. 6). When performed in the absence of hydroxyurea, the timing of anaphase was comparable for all three strains (not shown).

1.2f pds1-128 and rad23Δ ddi1Δ are sensitive to chronic gamma ray exposure

Wild type cells grow well on media with 0.1M hydroxyurea (Fig. 3). The same conditions are lethal for *pds1-128* and *rad23Δ ddi1Δ*. Neither mutant is particularly sensitive to short periods (6 h) on media containing non-arresting (0.1M) or arresting (0.4M) amounts of hydroxyurea (not shown): lethality comparable to that seen in the solid media assay is seen only after extended periods of growth (at least 24 h). One intriguing possibility is that, in addition to the extensively studied checkpoint controls which respond to accute DNA damage or replication blockage, distinct checkpoint mechanisms may be induced during chronic perturbations. If this were true, we would expect *pds1-128* and *rad23Δ ddi1Δ* to be sensitive to agents other than hydroxyurea, when delivered in a chronic manner. Therefore we tested whether *pds1-128* and *rad23Δ ddi1Δ* are sensitive to the continuous presence of a low amount of DNA damage. We found that both mutants are indeed sensitive to gamma irradiation delivered at a low dose rate (0.2 Gy/min for 15 hours; Fig. 7), even though *rad23Δ ddi1Δ* cells have a wild type sensitivity to high dose rate delivery of gamma rays.

1.2g pds1 and rad23 ddi1 mutations induce aneuploidy

Pds1p is essential for the inhibition of anaphase which occurs in response to checkpoint signals. Rad23p and Ddi1p are essential for a subset of these Pds1p-dependent checkpoint controls. The hypothesis central to this research predicts

that cells defective in these proteins should be prone to become aneuploid when challenged for efficient checkpoint control. To test this hypothesis, diploid cells were constructed that are homozygous for the *pds1-128* or *rad23Δ ddiΔ* mutations. Diploidy causes heterozygosity at the MAT (mating type) locus and thus renders such strains unable to mate. However, loss of heterozygosity at the MAT locus reinstates mating ability, therefore the generation of aneuploid cells can be assayed using a mating assay. In a pilot experiment, a/α WT, a/α *pds1-128/pds1-128*, and a/α *rad23Δ ddiΔ/rad23Δ ddiΔ* cells were grown for a defined number of cell doublings either in YEPD media or in YEPD media containing 0.1M hydroxyurea. Subsequently, a defined number of cells from each strain was tested for mating ability. Relative to background, aneuploid cells were not induced by the hydroxyurea treatment in the case of WT diploids. However, hydroxyurea increased the generation of cells capable of mating by 30% (+/- 5%) for a/α *pds1-128/pds1-128*, and a/α *rad23Δ ddiΔ/rad23Δ ddiΔ* Therefore, according to this preliminary data these mutants are prone to become aneuploid when replication is challenged.

2. Discussion

The genetic screens for elements which interact with the APC components Cdc16p and Cdc23p were largely unsuccessful; an exception was the isolation of *scs1-1*, a mutation which suppresses *cdc16-1*. The fruitfulness of the *pds1* screen (see below) provided a strong incentive to concentrate efforts in this area. However, a full characterisation of *scs1-1* will not be neglected.

The screen for proteins which interact with Pds1p, a protein essential for the regulation of chromosome segregation, was extremely successful. Three suppressors were identified: *RAD23*, *DDI1* and *ESP1*. This screen led to two important findings which are specifically relevant to the aim of this research. First, a novel Pds1p-dependent cell cycle checkpoint was discovered; this

checkpoint system coordinates replication with anaphase, preventing chromosome segregation when replication is incomplete. Loss of such a checkpoint control causes aneuploidy; *pds1* mutants display chromosome loss at an elevated rate. Second, Rad23p and Ddi1p were found to act upstream of Pds1p in this checkpoint pathway. *rad23 ddi1* mutants are checkpoint defective and suffer chromosome loss when challenged with replication inhibitors. Rad23p and Ddi1p are therefore important regulators of sister chromatid separation and contribute to the fidelity of chromosome segregation. The human homologues of *RAD23* and *DDI1* are structurally conserved, suggesting that these protein have analogous functions in human cell cycle control.

2.1 Detailed discussion of the *pds1* screen

2.1a Genetic interactions of PDS1 and RAD23/DDI1

In budding yeast Pds1p is an anaphase inhibitor. DNA damage, spindle perturbations and presumably replication inhibition, increase Pds1p stability, which in turn inhibits the onset of anaphase. The key role of Pds1p in M-phase checkpoint control has been well documented; less is known about factors regulating Pds1p in response to checkpoint signals. We have used a genetic screen to identify proteins which specifically interact with and regulate Pds1p.

High dosage *RAD23* and *DDI1* suppress the temperature sensitivity of *pds1-128* and rescue the hydroxyurea sensitivity of *pds1-128* (at the permissive temperature). Neither the temperature sensitivity nor the hydroxyurea sensitivity can be rescued *by RAD23/DDI1* over-expression in *pds1Δ* strains; i.e. there is no bypass of the cellular requirement for Pds1p. Phenotypic rescue of a null mutation by a dosage suppressor reveals that the suppressor can carry out, or compensate, the function of the protein which is absent. Suppressors that cannot bypass the null mutation must act upon, or be modulated by, the product in question (provided that the genetic interaction with the non-null mutation is

specific). The genetic interaction of *RAD23/DDI1* with *pds1* are of the latter class and determine that the products of these genes participate in a common pathway.

In agreement, high dosage *RAD23/DDI1* enhance the temperature sensitivity of *esp1* mutants: since Pds1p has an inhibitory effect on Esp1p at anaphase, this is precisely the expected outcome - stabilization of Pds1p should reduce the number of free *esp1* molecules, and therefore decrease the semi-permissive temperature for *esp1*^{ts} strains.

Given that Pds1p is known to be required for all of the known anaphase checkpoint control systems, and that *rad23Δ ddi1Δ* mutants are specifically defective in replication checkpoint control, we propose that Rad23p and Ddi1p act upstream of Pds1p.

2.1b pds1 and rad23Δ ddi1Δ mutants are defective in checkpoint control

pds1 mutants are hydroxyurea sensitive. This phenotype has been overlooked by others in the initial characterization of *pds1* checkpoint deficiencies. When synchronous cultures of *pds1-128* were released from G1 and grown in the presence of non-arresting doses of hydroxyurea, bud emergence, replication and the formation of G2 spindles occurred with the same timing as in wild type cells. However, spindle elongation was advanced in *pds1-128* mutants relative to the timing in wild type cells, demonstrating a defect in a checkpoint control which normally couples replication with mitotic progression. Rad23p and Ddi1p are also essential for this checkpoint response: in the above experiment, the behaviour of *rad23Δ ddi1Δ* mutants was comparable to that of *pds1-128*. Overexpression of *RAD23* or *DDI1* delayed anaphase when cells were grown in hydroxyurea, providing a mechanism to explain how high dosage *RAD23/DDI1* suppress *pds1* phenotypes. These experiments demonstrate the biological relevance of the genetic interactions between *RAD23/DDI1* and *PDS1*, and support the proposal that Rad23p and Ddi1p are required for a Pds1p-dependent replication

checkpoint.

2.1c Function of Rad23p and Ddi1p in checkpoint control

Rad23p is a highly conserved protein which has a nucleotide excision repair (NER) function; though not essential for DNA repair, Rad23p enhances the efficiency of NER. The repair function depends upon binding to the NER complex, via Rad4p (in yeast) or XPC (in human cells). In various experimental systems, much of the Rad23 protein, including the UBA domains, is dispensible for efficient NER. Further, the majority of cellular Rad23p is not associated with NER complexes. However, functions not related to DNA repair have not been assigned to Rad23p.

Almost nothing is known about the function of Ddi1p. *DDI1* has a UAS identical to that of *RAD23*, and is induced by DNA damage and hydroxyurea. Unlike *rad23* mutants, *ddi1* strains are not sensitive to ultraviolet light and are therefore not required for NER. Previous studies have shown that neither *rad23* nor *ddi1* mutants are defective in checkpoint control.

Here we describe unsuspected checkpoint functions of Rad23p and Ddi1p, not previously revealed due to the functional redundancy of these proteins in checkpoint control. The structural similarity of *RAD23* and *DDI1* supports this reasoning. A striking observation is the fact that Rad23p and Ddi1p both have UBAs - domains present in different classes of enzyme involved in ubiquitin-dependent proteolysis (11): this may prove to be relevant to the regulation of Pds1p degradation, a matter which will be investigated further. Rad23p and Ddi1p are likely to be universal checkpoint proteins; their homologues in mammals and other species are highly conserved, particularly within the UBA domains.

Since anaphase checkpoint control seems to depend on regulating Pds1p stability, it is logical that Rad23p/Ddi1p increase stability of Pds1p. Rad23p was

recently shown to interact physically with the complex responsible for Pds1p degradation: the M-phase specific 26S proteasome (12). This may provide a mechanism through which the anaphase checkpoint may be executed. Rad23p and Ddi1p may directly regulate proteasome function resulting in increased Pds1p stability.

2.1d Model for coordination between replication completion and mitosis

Fig. 8 presents a model describing how regulated Pds1p stability controls the metaphase-anaphase transition. In G1 and early S phase, cells neither have a spindle nor replicated chrmosomes: there is no need to inhibit anaphase - cells have no Pds1p. Pds1p production in S phase prevents separation of newly replicated chromosomes and inhibits elongation of new G2 spindles: Pds1p remains stable because APC activation requires high Clb1p/Clb2p-associated kinase activity. Whether or not replication is perturbed, Cdc28p-Clb1p/Clb2p kinase activity peaks in a timely fashion, causing APC activation and Pds1p ubiquitination. We propose that Rad23p and Ddi1p become essential if the APC is activited before replication is completed: either protein would bind to the proteasome, inhibiting proteasome activity, and thus prevent Pds1p degradation.

Pds1p degradation allows Esp1p-mediated displacement of Scc1p from chromosomes, Scc1p being the putative "glue protein" which prevents sister chromatid separation (6,7). In this model, a Pds1p-Esp1p complex exists before anaphase, Pds1p being an Esp1p inhibitor. We have shown that *pds1-128* is suppressed by increased dosage of *ESP1*: the above model is not consistent with our genetic data unless we assign an essential pre-anaphase function to the Pds1p-Esp1p complex: since *pds1Δ* can also be suppressed by high dosage *ESP1*, this early function can be carried out or compensated by an increased amount of Esp1p.

Pds1p is essential for a checkpoint control which responds to replication

inhibition. We propose that in response to replication inhibition, Rad23p and Ddi1p mediate checkpoint signals which prevent Pds1p proteolysis. Rad23p and Ddi1p may participate in novel checkpoint systems which respond to chronic, but not accute, exposure to agents which damage DNA or perturb replicative DNA synthesis.

2.2 Relevance to the original hypothesis

Our hypothesis is that chromatid cohesion proteins and proteins involved in their regulation will be defective in people more prone to develop breast cancer and other diseases in which aneuploidy is a key contributing factor. The goal of this research is directly relevant to the study of breast cancer because the aim is to determine which proteins ensure high fidelity chromosome segregation during anaphase. We have identified and partly characterized 2 such proteins and find that mutations in these regulators contributes to the induction of aneuploidy.

3. Experimental Methods, Assumptions and Procedures

3.1 Yeast strains, media and growth conditions

All strains are derivatives of BF264-15DU *MATa ade1 his2 leu2-3,112 trp1-1a ura3 Dns* (14). Relevant genotypes are described below. Cultures were grown at 30°C, unless otherwise stated, in YEP media containing 2% dextrose (YEPD), raffinose (YEPR) or galactose (YEPG) as a carbon source. Strains were constructed according to standard genetic procedures (15) except for gene disruptions which were performed as described by Wach et al. 1994 (16).

3.1a Genotypes of specific yeast strains

DCY1180	*MAT**a** bar1Δ*
DCY1167	*MATα pds1-128*
DCY1283	*MAT**a** bar1Δ rad23::KAN^R ddi1::KAN^R*
DCY1270	*MAT**a** bar1Δ GAL1:RAD23(LEU2)*
DCY1278	*MAT**a** bar1Δ GAL1:DDI1(LEU2)*

DCY1228	*MATa bar1Δ pds1::KAN^R*
DCY1234	*MATa bar1Δ pds1::KAN^R YEp-ESP1(URA3)*
DCY1235	*MATa bar1Δ pds1::KAN^R YEp-RAD23(URA3)*
DCY1236	*MATa bar1Δ pds1::KAN^R YEp-PDS1(URA3)*
DCY1237	*MATa bar1Δ pds1::KAN^R YEp-DDI1(URA3)*
DCY1233	*MATa bar1Δ pds1::KAN^R YEp24(URA3)*
DCY1195	*MATa pds1-128 YEp-DDI1(URA3)*
DCY1196	*MATa pds1-128 YEp-ESP1(URA3)*
DCY1199	*MATa pds1-128 YEp-RAD23(URA3)*
DCY1210	*MATa pds1-128 YEp-PDS1(URA3)*
DCY1213	*MATa pds1-128 YEp24*
DCY1384	*MATa esp1::KAN^R YCp-esp1-N5(TRP1) GAL1:RAD23(LEU2)*
DCY1386	*MATa esp1::KAN^R YCp-esp1-B7(TRP1) GAL1:RAD23(LEU2)*
DCY1668	*MATa esp1::KAN^R YCp-esp1-B7(TRP1) GAL1:DDI1(LEU2)*
DCY1264	*MATα bar1Δ rad23::KAN^R*
DCY1258	*MATα bar1Δ ddi1::KAN^R*
DCY1359	*MATα rad9::TRP1*
DCY1407	*MATa pds1-128 GAL1:DDI1(LEU2)*
DCY1272	*MATa bar1Δ pds1-128 GAL1:RAD23(LEU2)*
DCY1277	*MATa bar1Δ pds1:KAN^R GAL1:DDI1(LEU2)*
DCY1636	*MATa pds1:KAN^R GAL1:RAD23(LEU2)*

3.2 Screen for mutations which bypass the metaphase arrest of *cdc23-1*

This screen is designed to isolate temperature-sensitive mutations which allow chromatid separation at the non-permissive temperature in the *cdc23-1* mutant. 20, 000 *cdc23-1* cells were mutagenized then arrested in metaphase at the non-permissive-temperature for 2 hours. Nocodazole (15 μg/ml) was added and the culture shifted back to the permissive-temperature. *cdc23-1* remain in metaphase following this synchrony due to the metaphase sensitivity to nocodazole which activates the spindle checkpoint (in non-mutated controls 99% of the cells arrested in metaphase). However, telophase arrested cells complete division: cycle progression is not blocked by nocodazole and the shift to the permissive temperature restores Cdc23p activity allowing mitotic exit. Such double mutants (proceeding through G1) were isolated according to size by centrifugal elutriation. The phenotypes of several mutants derived from this screen were analysed. Mutants were organized into complementation groups by

standard procedures (14), then one from each class was examined in detail by studying the terminal phenotype at the non-permissive temperature.

3.3 Screen for *cdc16-1* mutational enhancers and suppressors

For isolating mutations which suppress/enhance the temperature-sensitivity of *cdc16-1,* 20, 000 UV mutagenized *cdc16-1* cells (irradiated to give 50% lethality) were either (1) selected for growth at the non-permissive temperature for *cdc16-1* (supressors) or (2) tested for lethality at the semi-permissive temperature for *cdc16-1* (enhancers). A single mutant allele was isolated which confers suppression of the *cdc16-1* temperature-sensitivity. This allele was outcrossed from the *cdc-16* allele and analysed further according to standard genetic procedures (15).

3.4 Screens for dosage suppressors

Screens to isolate dosage suppressors of *pds1-128, cdc16-1* or *cdc23-1* were carried out as follows. Strains were transformed with a 2μ genomic library (17) then clones tested for suppression of temperature-sensitivity. For pilot attempts, about 20, 000 clones were analysed by standard precedures to isolate suppressors showing plamid-dependent rescue (17).

3.4b Isolation of pds1-128 dosage suppressors

pds1-128 was originally isolated in a screen designed to identify mutational enhancers of cyclin *CLB2* over-expression (8). *pds1-128,* and *pds1-128/GAL1:CLB2* cells grow poorly at 37°C on YEP dextrose media and are inviable at 37°C on YEP galactose. Characterization of *pds1-128* revealed phenotypes consistent with those of the previously isolated *pds1-1* allele (3), except that *pds-128* is highly sensitive to hydroxyurea. *pds1-128* suppressors were isolated by transformation with a *Saccharomyces cerevisiae* genomic library as

described above. Transformants were replicated on to YEPG plates and suppressors selected at 37°C. In excess of 160,000 independent transformants were analysed to identify 110 colonies rescued in a plasmid-dependent manner. 80/110 colonies appeared wild type at 37°C on YEPG media; 13 of these clones were examined further, and all of these isolates contained *PDS1*. 30/110 colonies grew more poorly than wild type at 37°C on YEPG media. Plasmids isolated from each of these were subcloned by standard molecular biology procedures and the relevant ORF's identified by re-transformation into *pds1-128* cells. Dosage suppression was confirred by *ESP1*, *RAD23*, or *DDI1* (5 plasmids contained none of these genes and did not rescue *pds1-128* upon retransformation).

3.5 General cell and molecular biology procedures

Samples were prepared for FACScan analysis as described (18). Nuclei were stained with DAPI or PI as described (19). Cells were stained with tubulin antibodies as described by Mondesert and Reed, 1992 (19). Mutation of *DAM1* destruction boxes was performed using a Clontech mutagenesis kit, Clontech Inc., CA. For spindle elongation/nuclear division assays, strains were grown to saturation in YEPR then diluted to OD 0.15. G1 arrest with 100-200 ng/ml alpha factor was complete within 2.5 hours. Cells were washed several times and innoculated in YEPD or YEPG containing 0.1M hydroxyurea at 30°C. At intervals, samples were fixed with 3.7% formaldehyde solution for 1 hour, then spindle elongation scored by visualizing a Tub1-GFP fusion protein by direct fluorescence microscopy (20). FACScan samples were prepared in parallel and used to score nuclear division (18).

3.7 Drug and radiation sensitivity assays

Hydroxyurea sensitivity assays were performed in two ways. (i) log-phase cultures were innoculted in liquid YEPD containing 0.4M or 0.1M hydroxyurea

(or no HU for control) for 6-24 hours then washed X3 in YEPD and specific numbers of cells plated on YEPD plates. Surviving colonies were counted after 2-3 days growth and the colony forming ability calculated relative to control colony survival. (ii) log-phase cultures were spotted in serial dilution on YEPD plates containing 0, 0.05, 0.075, 0.1, 0.15 and 0.2M hydroxyurea. After 24 hours, microcolonies were scored by counting at least 500 colonies per sample. Spot growth was recorded after 2-3 days. For nocodazole sensitivity assays, log-phase cultures were innoculted in liquid YEPD containing 0.15 μg/ml nocodazole and shaken for 6 hours at 30°C then washed X3 in YEPD and specific numbers of cells plated on YEPD plates. Surviving colonies were counted after 2-3 days growth and the colony forming ability calculated relative to control colony survival. For UV and gamma ray sensitivity assays, log-phase cultures were spotted in serial dilution on YEPD plates then irradiated. Spot growth and colony formation was recorded after 2-3 days.

3.8 Mating assay for estimating induced aneuploidy

a/α WT, *pds1-128/pds1-128*, and *rad23Δ ddiΔ/rad23Δ ddiΔ* cells were grown for a defined number of cell doublings either in YEPD media or in YEPD media containing 0.1M hydroxyurea. Subsequently, a defined number of cells from each strain was mixed with an excess of an a or α mating type 'tester' strain and incubated at 25°C to allow mating to occur. These mating mixtures were plated on selective media which only allows growth of diploids formed from a mating of a 'tester' cell and one of the WT, *pds1-128/pds1-128*, or *rad23Δ ddiΔ/rad23Δ ddiΔ* cells.

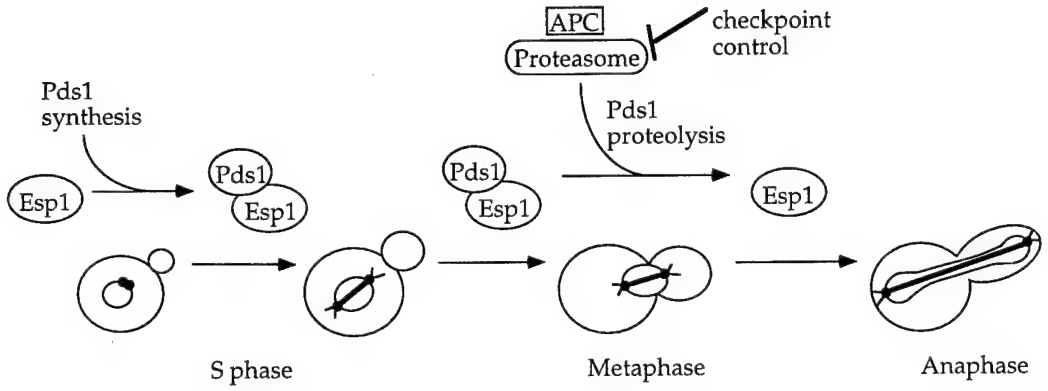

Fig1

Model for Pds1/Esp1 cell cycle functions. During early S phase newly synthesized Pds1p associates with Esp1p allowing correct formation of G2 spindles. At metaphase Pds1p is ubiquitinated and degraded by the APC and proteasome particles releasing Esp1p which promotes the onset of anaphase. Checkpoint control can act at various levels by preventing Pds1p proteolysis

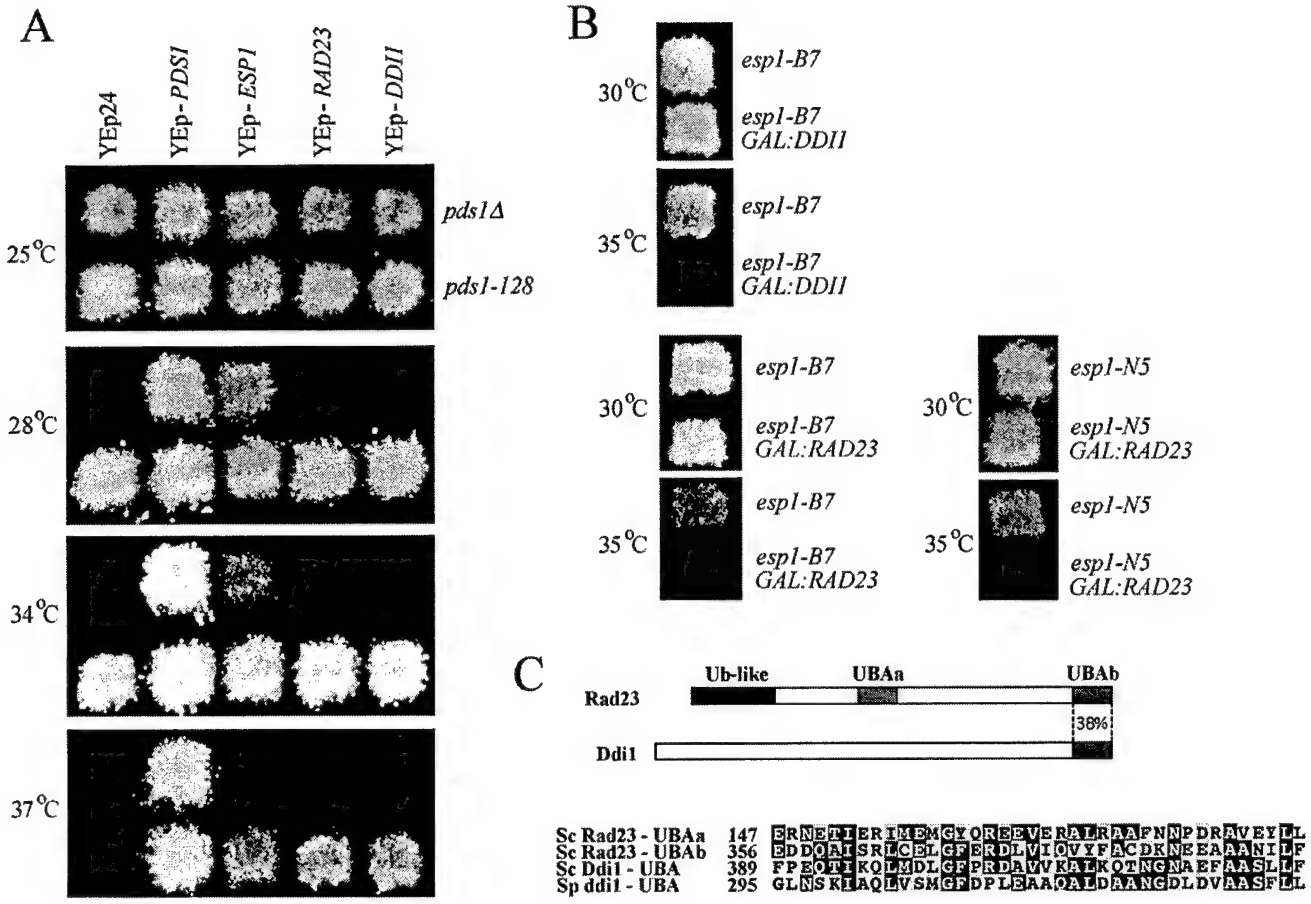

Fig 2

A. Dosage suppressers of *pds1*. Comparison of suppression of *pds1-128* and *pds1Δ* temperture sensitivity by multi-copy plasmids YEp-*PDS1*, YEp-*RAD23*, YEp-*DDI1* and YEp-*ESP1*. Patches were grown at 25°C on synthetic media containing dextrose, but without uracil to prevent loss of the YEp24-based plasmids. These patches were replicated on to YEP dextrose plates and grown at 25°C - 37°C for 2 days.

B. Genetic interaction between *RAD23/DDI1* and *esp1*. Strains carrying temperature sensitive *esp1* alleles (*esp1-N5* and *esp1-B7)* with or without RAD23/DDI1 alleles under control of the strongly inducible *GAL1* promotor, were grown at 30°C on YEP dextrose plates then replicated to YEP galactose plates and grown for 2 days at 30°C-35°C.

C. Structural similarity between *RAD23* and *DDI1* genes. Upper panel; box diagram showing relative sizes of each ORF and the size and position of UBA and ubiquitin-like domains. C-terminal UBAs, right hand side, share 38% identity. Lower panel; alignment of UBA domains. Numbers to the left of the alignments show the position in the protein of the first UBA amino acid. Sp ddi1 shows the UBA region of a hypothetical fission yeast protein (gb | Z69728, gi | 1204230) which has an over all 32% identity to budding yeast Ddi1p. Probable Ddi1p homologues giving high identity scores are present in H. sapiens (gb | AA406136, gi | 2064117; dbest data base), C. elegans (gb | U50068, gi | 1208859) and L. major (gb | AC002305, gi | 2429118).

Table 1

	U.V. light[1]	γ rays[2]	Hydroxyurea[3]	Nocodazole[4]
pds1-128	-	✓✓	✓✓✓	✓✓✓
rad23Δddi1Δ	✓	-	✓✓✓	-
rad23Δ	✓	-	✓	-
ddi1Δ	-	-	-	-
rad9Δ	✓✓✓	✓✓✓	-	-

[1] 10-100 Joules/m^2; delivered over 10 seconds maximum
[2] 50-200 Gy delivered at 3.6 Gy/min
[3] Sensitivity tested in two ways: (1) strains spotted onto YEP dextrose plates containing 0.075-0.2 M hydroxyurea or (2) strains grown in liquid YEP dextrose media with 0.1 or 0.4 M hydroxyurea for 24 hours then colony survival scored on YEP dextrose plates
[4] 3 hour treatment with 15 μg/ml

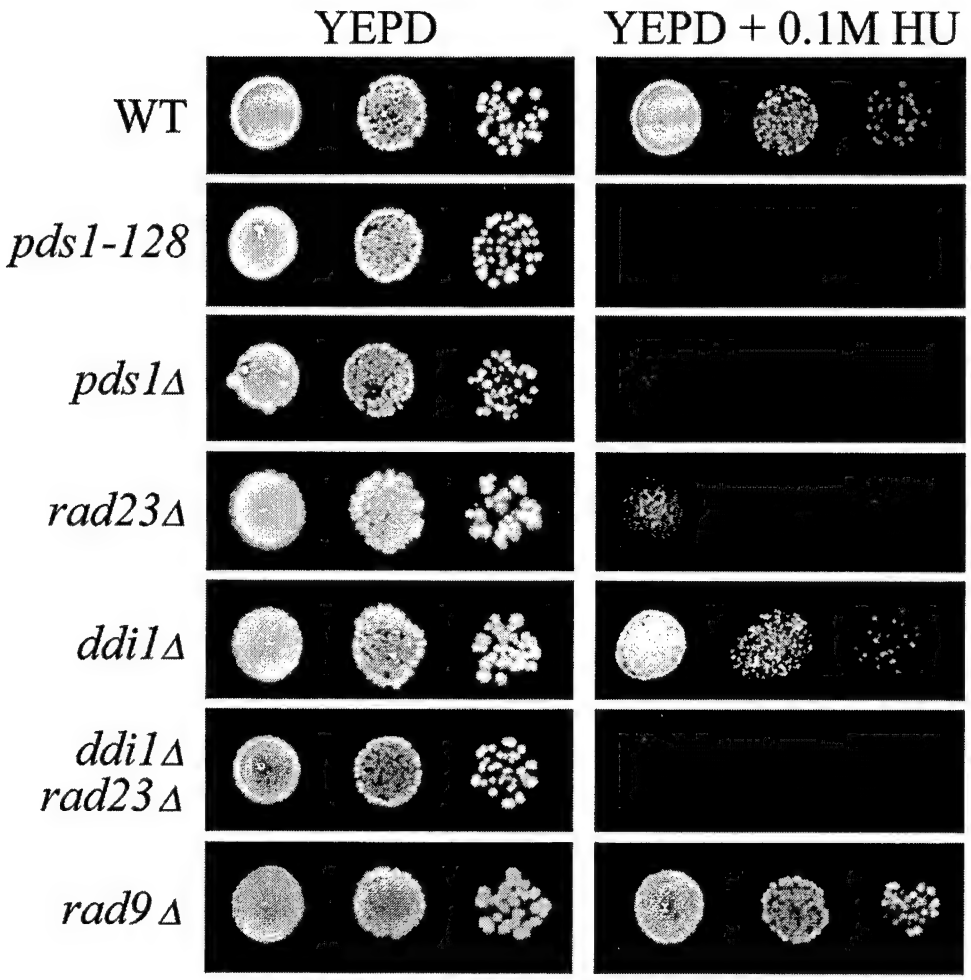

Fig 3

Sensitivity of *pds1* and *rad23Δ ddi1Δ* mutants to hydroxyurea. Strains were grown to mid-log phase then serial dilutions spotted on to YEPD plates or YEPD plates containing hydroxyurea and grown for 2-3 days at 30°C (25°C for *pds1Δ*).

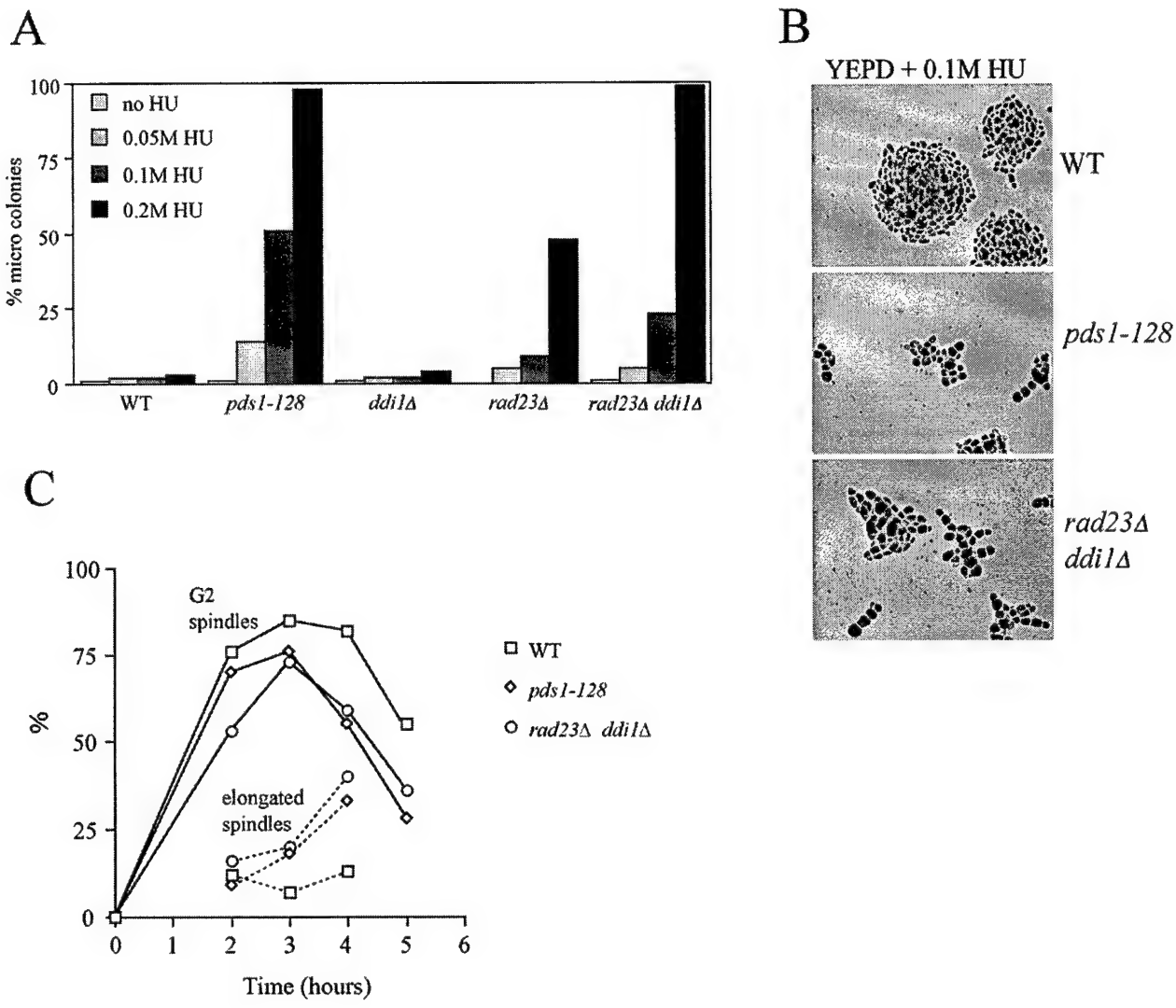

Fig 4

Checkpoint defect in *pds1* and *rad23Δ ddi1Δ* mutants. A,B. Wild type, *pds1-128* and *rad23Δ ddi1Δ* strains were grown on solid YEPD media containing hydroxyurea: quantification of microcolony formation (A) and images of microcolonies (B). C. Timing of budding, G2 spindle formation and spindle elongation in wild type and mutant cells grown in liquid YEPD containing 0.1M hydroxyurea following release from G1.

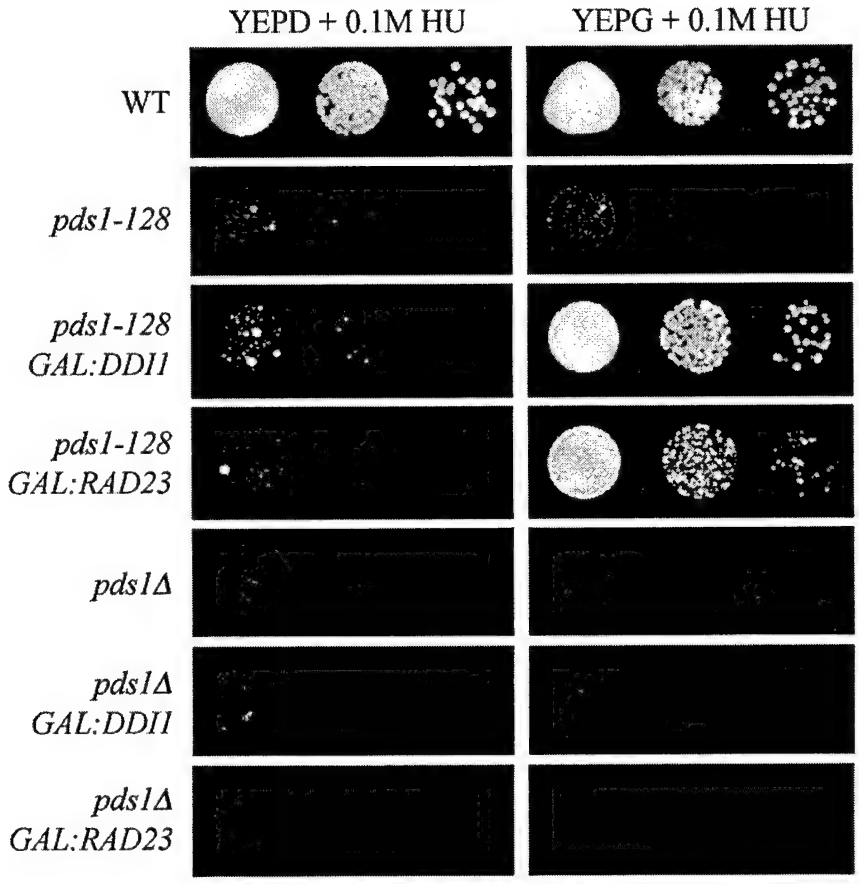

Fig 5

Suppression of *pds1-128* hydroxyurea sensitivity by *RAD23/DDI1* overexpression. Strains were spotted (as described for Fig. 2) on to YEPD/YEPG plates containing hydroxyurea to repress or induce *GAL1:RAD23* and *GAL1:DDI1*.

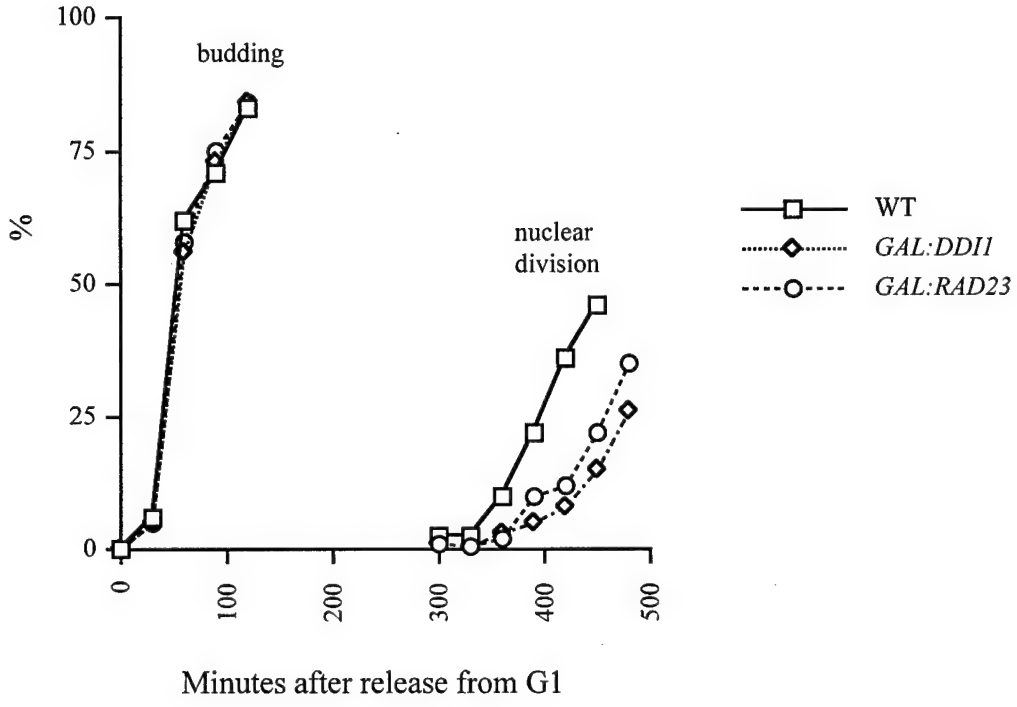

Fig 6
Delay of nuclear division when *DDI1* or *RAD23* are overexpressed. Cells were synchronized in G1 and released in to media containing hydroxyurea as described in experimental procedures. y-axis shows % of budded cells (for budding) and % of budded cells with 2 nuclei (for nuclear division). This graph shows data representative of several experiments: nuclear division was consistently delayed by at least 40 minutes when *DDI1* or *RAD23* were over-expressed.

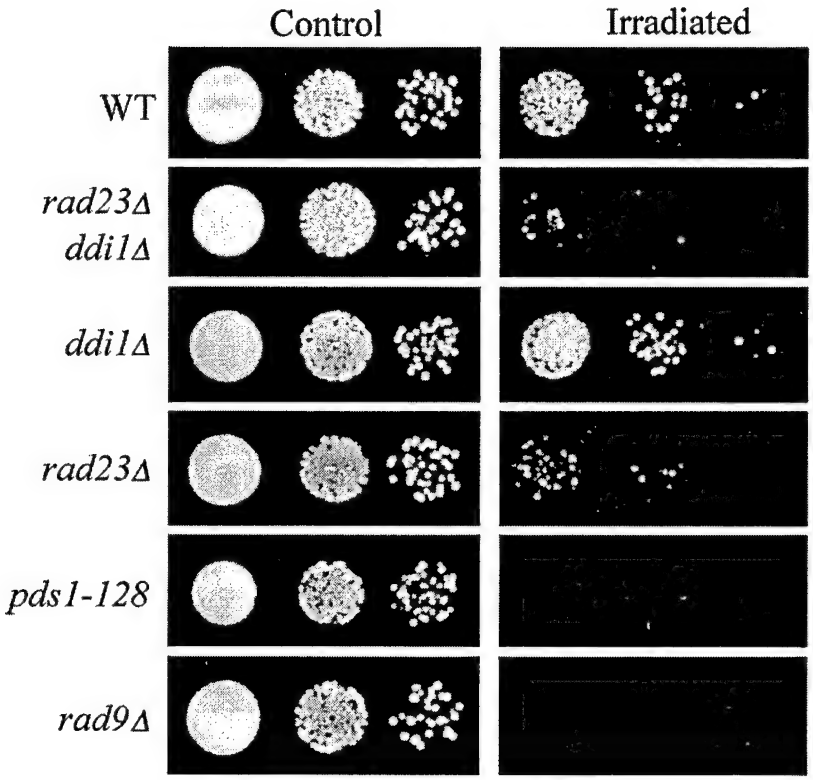

Fig 7

Sensitivity of *pds1* and *rad23Δ ddiΔ* mutants to chronic gamma ray exposure. Strains were spotted on to YEPD plates as described for Fig 2, irradiated for 15 hours at 0.036 Gy per minute, then grown for 1-2 days at 30°C.

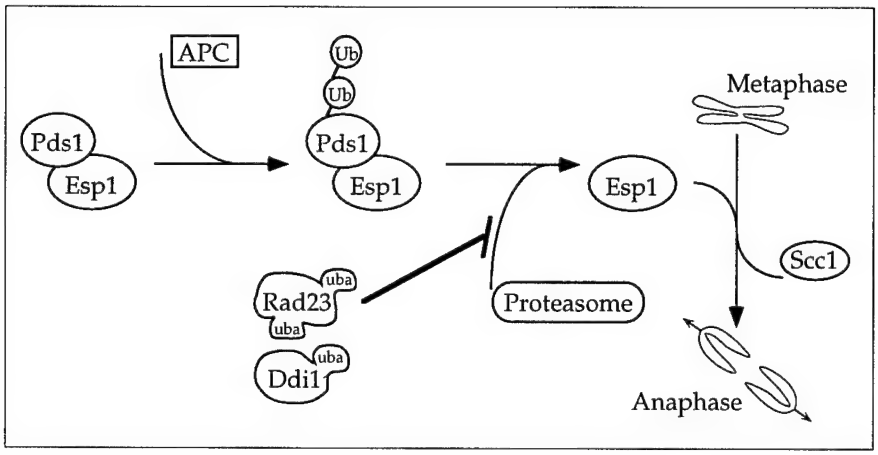

Fig 8

Model for checkpoint control by Rad23p and Ddi1p. Checkpoint signals induced by replication inhibition activates Rad23p and Ddi1p which prevent Pds1p proteolysis. Mode of action of Rad23p is likely to be through inhibition of proteasome activity since Rad23p physically interacts with proteasome subunits

Conclusions

Summary

Aberrant mitotic segregation of chromosomes causes aneuploidy which is a key event in the etiology of breast cancer: our goal is to identify and characterize new proteins which regulate chromosome segregation. Using yeast genetics as an experimental system, we have established several productive strategies for isolating such proteins. One such procedure revealed strong genetic interactions between *RAD23*, *DDI1*, *ESP1* and the anaphase regulator *PDS1*. These genetic data formed a basis for a series of experiments from which we determined that Rad23p and Ddi1p are essential for a Pds1p-dependent checkpoint system that couples replication with mitosis: mutant *rad23 ddi1* cells become aneuploid when challenged with replication inhibitors. We have therefore identified 2 proteins required for the regulation of chromosome segregation in yeast. Both *RAD23* and *DDI1* are highly conserved in human cells, though defects in these genes have not been previously implicated in the generation of aneuploid cells.

Implications

The central hypothesis on which this work is based is as follows: proteins involved in regulating chromosome segregation will be defective in people more prone to develop breast cancer and other diseases in which aneuploidy is a key contributing factor. Our research approach makes two assumptions which have been borne out conclusively by the first year of the fellowship.

First, we set out to use powerful genetic approaches in yeast to rapidly identify the proteins important for regulated chromosome segregation: the relevance of this approach was based on the fact that the proteins known to be involved in the process of chromosome segregation have a high degree of

evolutionary conservation. The 2 genes that we identified so far have very closely related human homologues, demonstrating that our system is indeed highly relevant.

Second, we assumed that defects in proteins that regulate chromosome segregation would cause aneuploidy. The first 2 proteins we have identified and characterized fullfill this criteria exactly as predicted.

It follows that defects in such proteins may cause reduced segregation efficiency in humans and therefore increase the frequency of aneuploidy in dividing cells of the breast epithelium. An ability to recognize such defects would have a vast impact on our capacity to identify individuals prone to acquiring aneuploid cells. Clearly, a detailed understanding of how segregation regulators operate is extremely desirable. This knowledge will eventually allow us to define genetic and environmental factors which cause aneuploidy; many of these factors will be critically involved in the etiology of breast cancer.

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